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COMPARATIVE BIOCHEMISTRY AND METABOLISM

Part II: Naphthalene Lung Toxicity

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals, "Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

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FOR THE COMMANDER

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Earlier studies have shown that intraperitoneal administration of naphthalene causes selective pulmonary bronchiolar necrosis in mice and have suggested a role for reactive naphthalene metabolites in the tissue damage. These studies have been extended to show that covalent binding of reactive naphthalene metabolites to tissue macromolecules in lung, liver and kidney is dose-dependent. High levels of covalent binding occur only after tissue glutathione levels are depleted substantially and only at doses of 200 mg/kg

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and above. The 200 mg/kg dose is the lowest dose at which lung lesions are observed. Piperonyl butoxide pretreatment decreases pulmonary damage and covalent binding and partially blocks glutathione depletion by 400 mg/kg naphthalene. In comparision, SKF 525 A pretreatment decreased covalent binding levels to a much smaller extent and failed to alter glutathione depletion or pulmonary damage after 400 mg/kg naphthalene. When compared to vehicle pretreated controls, phenobarbital or 3-methylcholanthrene pretreatment produced no change in the severity of lung damage after doses of naphthalene ranging from 50 to 400 mg/kg nor did these inducers alter covalent binding at doses of naphthalene of 200 mg/kg or less. Pretreatment of mice with p-xylene preferentially inactivated pulmonary cytochrome P450 and blocked naphthalene induced lung damage but failed to selectively decrease covalent binding of reactive metabolites in the lung. Current studies are continuing to examine the role of cytochrome P450 dependent metabolic activation of naphthalene in the lung in the bronchiolar damage.

PREFACE

This is the annual report of the subprogram on Comparative Biochemistry and Metabolism, Part II: Naphthalene Lung Toxicity and concerns work performed by the Department of Community and Environmental Medicine of the University of California, Irvine, on behalf of the Air Force under contract #F33615-80-C-0512, Work Unit 63020115. This document describes the accomplishments of the subprogram from June 1980 through May 1981. K.C. Back, Ph.D., Chief of Toxicology Branch, was the technical monitor for the Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, Ohio.

A.R. Buckpitt served as coordinator of Part II of the subprogram. Acknowledgment is made to two undergraduate students, Darren L. Warren and David L. Brown, Jr., for their significant research contributions.

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INTRODUCTION

This report summarizes the work conducted from June 1980 through May 1981 in the subprogram on Comparative Biochemistry and Metabolism, Part II: Naphthalene Lung Damage. This section of the subprogram was established in an attempt to understand on a molecular level the sequence of biochemical steps critical to pulmonary damage induced by certain chemicals. In this section of the report, the relevance of such studies to USAF/USN concerns will be outlined along with the literature background on the specific chemical under study.

Study of the biochemical mechanisms of lung damage caused by naphthalene was felt to be appropriate from several standpoints. This aromatic hydrocarbon is of direct interest to the USAF/USN because of its presence in and structural similarity to many of the hydrocarbons in lubricants and fuels including shale oil (Guerin et al., 1978; Fruchter et al., 1978) and because it serves as a starting material in the synthesis of both decalin and tetrahydronaphthalene. Intraperitoneal administration of naphthalene causes damage to bronchiolar epithelial cells of mice and hamsters. Considerable evidence now suggests that non-ciliated bronchiolar epithelial cells (Clara cells) are a major locus of pulmonary cytochrome P-450 monooxygenase activity and thus may be important target cells in the lung for chemicals requiring metabolic activation (see Boyd, 1980, for review). Since the bioactivation of chemically inert environmental agents may play a role in the pathogenesis of lung diseases from cancer to emphysema and fibrosis, it is important to thoroughly understand the interplay of activating and detoxifying enzymes in the lung and factors which may affect the critical balance of these two processes.

A further area of concern not only to USAF/USN toxicologists but to the scientific community in general is the appropriateness of various animal species as a model for the human. The mouse appears to be considerably more sensitive to lung damage by a variety of metabolically activated xenobiotics than are other rodent species. Pulmonary damage is elicited in the mouse but not the rat by systemic administration of bromobenzene (Reid et al., 1973), naphthalene (Mahavi et al., 1977), butylated hydroxytoluene (Adamson et al., 1977) and 3-methylfuran (Boyd et al., 1978). Likewise, administration of 3-methylindole, a toxicant requiring metabolic activation, results in severe pulmonary damage in cattle, sheep and goats but not rodents (Bray and Carlson, 1979; Hammond et al., 1980). Whether these observed species differences are the result of differences in the pulmonary enzymes that bioactivate or detoxify

such compounds or whether such differences in sensitivity are simply due to altered disposition of the compound in sensitive vs. non-sensitive species is at this point largely unknown. While substrate specificities of rodent lung xenobiotic metabolizing enzymes have been studied extensively and in some cases the enzymes have been purified to homogeneity (Wolf et al., 1978, 1979), little has been done to characterize xenobiotic metabolizing enzymes in human lung tissue. With few exceptions, studies in human tissue have focused on the metabolism of benzo(a)pyrene (Prough et al., 1977, 1979; McManus et al., 1980); nothing has been reported concerning the ability of human lung tissue to catalyze the metabolic activation of chemicals known to cause pulmonary damage in rodents. Thus, one of the long-term goals of the research undertaken in this subprogram is to develop appropriate in vitro methods for studying those steps critical to the pulmonary damage by naphthalene in animals which can be applied to human lung tissue. The importance of such studies stems both from the information they provide concerning the risk of exposure of humans to hydrocarbons like naphthalene and in more general terms the information they yield about which, if any, laboratory rodents are good animal models for studies of metabolically activated pulmonary toxicants.

BACKGROUND

BRONCHIOLAR DAMAGE

The pulmonary bronchiolar necrosis caused by intraperitoneal administration of various aromatic hydrocarbons, including naphthalene, was first reported by Reid et al. (1973). Subsequent examination of the lungs of mice by both light and electron microscopy demonstrated that ip administration of naphthalene induced selective necrosis of the non-ciliated bronchiolar epithelial cells (Clara cells). Naphthalene-induced bronchiolar damage was time-dependent (maximal at 12-24 hours) and dose-dependent. These results were confirmed by this laboratory and were reported in last year's annual report (Shank et al., 1980). In addition, pretreating mice with piperonyl butoxide, an inhibitor of cytochrome P-450 monooxygenases, or with diethylmaleate, a depletor of tissue glutathione, was found to block or enhance respectively the bronchiolar damage of subsequently administered naphthalene. These results suggested that a metabolite or metabolites of naphthalene, rather than the parent compound, was involved in the tissue necrosis and that glutathione played a major role in the detoxification of such a metabolite(s).

Two recent studies also have indicated that close structural analogs of naphthalene, 1-nitronaphthalene and 2-methylnaphthalene cause extensive lung damage (Dankovic and Cornish, 1981; Griffith et al., 1981). In contrast to our results with naphthalene, Franklin et al. (personal communication) have been unable to demonstrate the involvement of cytochrome P-450 monooxygenases in 2-methylnaphthalene induced pulmonary damage.

METABOLISM

Metabolites of naphthalene including 1-naphthol, naphthalene-1,2-oxide, naphthalene-1,2-dihydrodiol and various sulfate, glucuronide and glutathione conjugates derived therefrom have been isolated and identified (see Jerina and Daly, 1974, for review). More recent studies on the metabolism of this hydrocarbon in rats has revealed the presence of urinary metabolites which are derived from dihydrodiol epoxide and diepoxide precursors (Horning et al. 1980). The chemical reactivity of such metabolites and the finding that a dihydrodiol epoxide metabolite of benzo(a)pyrene is the ultimate carcinogenic metabolite formed from this polycyclic aromatic hydrocarbon (Weinstein et al., 1976) suggests that both the diol epoxide and diepoxide metabolites of naphthalene may be toxicologically important. Not unexpectedly, the acute toxicity of both epoxides is considerably greater than the parent hydrocarbon (Horning et al., 1980).

In vitro, naphthalene is metabolized by rat liver microsomes to derivative(s) which bind covalently to microsomal protein. As appears to be the case with other aromatic hydrocarbons like bromobenzene (Hesse et al., 1980), reactive metabolites of naphthalene appear to arise from secondary metabolism of 1-naphthol and not from the parent hydrocarbon itself (Hesse and Metzger, 1979). The addition of ascorbic acid or superoxide dismutase to the incubation did not decrease the covalent binding thereby suggesting that reactive quinone or semiquinone metabolites of naphthalene are not involved in the binding process.

In vivo studies described in last year's annual report (Shank et al., 1980) demonstrated that radiolabel from the ip administration of 200 mg/kg ^{14}C-naphthalene was bound covalently to tissue macromolecules. Binding was maximal in lung, liver and kidney (tissues with relatively high cytochrome P-450 monooxygenase activity) and was low in tissues such as muscle which lacks detectable P-450 activity. The covalent binding was time-dependent and preceded the development of tissue lesions. Hepatic and pulmonary levels of reduced glutathione decreased rapidly after

administration of 200 mg/kg naphthalene; the decrease in tissue glutathione levels correlated well with the increase in covalent binding. Pulmonary, hepatic and renal covalent binding from 200 mg/kg ^{14}C-naphthalene was increased markedly by diethyl maleate pretreatment but was unaffected by piperonyl butoxide pretreatment.

RELATIONSHIP OF REACTIVE METABOLITE FORMATION TO LUNG DAMAGE

While the data cited above suggested the possible involvement of reactive naphthalene metabolites in bronchiolar necrosis induced by this hydrocarbon, there were several inconsistencies. Histopathology studies indicated that naphthalene caused selective damage in the lung. Hepatic or renal lesions were not observed at any dose tested in the mouse. Yet higher levels of covalent binding (calculated on a per mg protein basis) were found in liver and kidney than in lung. In addition, pretreatment with piperonyl butoxide blocked the pulmonary damage of 300 or 500 mg/kg doses of naphthalene yet failed to significantly decrease covalent binding levels in lung, liver, or kidney after 200 mg/kg ^{14}C-naphthalene. Thus, further studies were initiated to determine whether the formation of chemically reactive naphthalene metabolites, which covalently bind to tissue macromolecules in the lung and cause depletion of glutathione, play a role in lung damage.

An additional question which required attention was whether metabolism of naphthalene by pulmonary monooxygenases is essential to the formation of those metabolites which are bound and/or are toxic in the lung. In this regard, three possibilities must be considered and these are outlined in Figure 1. As is the case with the pulmonary bronchiolar cytotoxin 4-ipomeanol (Boyd and Burka, 1978), naphthalene may be metabolized in situ in the lung to a metabolite(s) which damages the cells of the bronchiolar epithelium. Another possibility is that the liver is forming a stable metabolite(s) of naphthalene such as 1-naphthol which circulates to the lung and undergoes further metabolism in pulmonary tissue to a derivative(s) involved in the lung damage. The third potential pathway is the formation of reactive naphthalene metabolite(s) in the liver which is sufficiently stable to circulate to the lung and cause damage to pulmonary cells. Precedent for such a mechanism comes from research on lung damage by the pyrrolizidine alkaloid, monocrotalin. Monocrotalin has been shown to undergo metabolic activation in the liver to reactive pyrrolic metabolites which subsequently circulate, covalently bind to cellular macromolecules and damage capillary endothelial cells of the lung. (See review by Huxtable, 1979.) Thus, before comparative in vitro metabolism studies can be initiated, in vivo studies addressing the

SCHEMATIC DIAGRAM SHOWING POSSIBLE INVOLVEMENT OF THE LIVER IN THE METABOLIC ACTIVATION OF NAPTHALENE

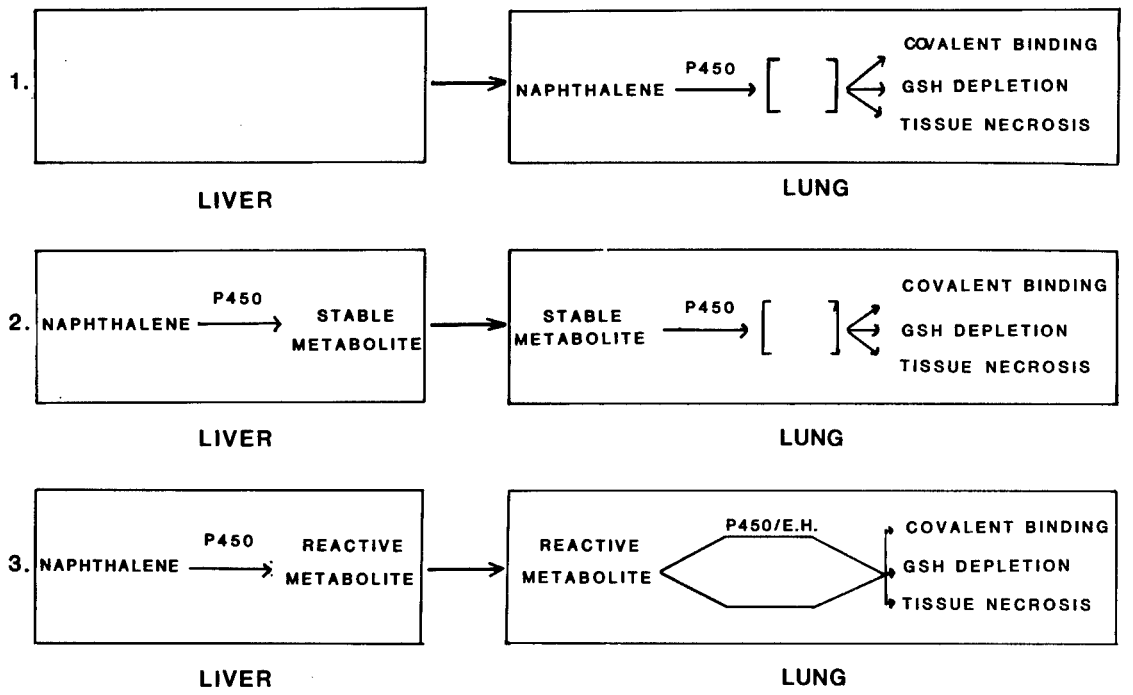


Figure 1. Schematic diagram showing possible involvement of the liver in the metabolic activation of naphthalene

questions of the role of reactive metabolites in lung damage and the source of the lung damaging metabolites must be completed. The experiments described in this report and those continuing in the laboratory are designed to answer these two fundamental questions.

RESEARCH PROGRAM

RELATIONSHIP OF FORMATION AND FATE OF REACTIVE NAPHTHALENE METABOLITE(S) AND PULMONARY DAMAGE

Dose Response Covalent Binding/Glutathione Depletion

Studies reported in the 1980 annual report had shown that the pulmonary damage by naphthalene was dose-dependent. If a positive correlation between pulmonary damage by naphthalene and the covalent

binding of reactive metabolites from this hydrocarbon in the lung was to be demonstrated, covalent binding of reactive metabolites in the lung would have to be dose-dependent as well. Thus, groups of 3-4 male Swiss Webster mice (25-30 g, obtained from Simonsen Breeding Labs, Gilroy, CA) were treated intraperitoneally with 1-[^{14}C]-naphthalene (Amersham Searle, Arlington Heights, IL/ >99.8% chemical and radiochemical purity by reverse phase high pressure liquid chromatography) at doses ranging from 25 to 600 mg/kg. The specific activities of the dose solutions were: 1359 dpm/nmole (25,50 mg/kg); 471 dpm/nmole (100, 200 mg/kg); and 117 dpm/nmole (400, 600 mg/kg). Naphthalene was dissolved in corn oil such that 0.1 ml was administered per 10 g body weight. Animals were sacrificed by decapitation after dosing and lungs were perfused thoroughly with ice cold heparinized saline. Lung, liver, kidney and muscle were removed quickly and frozen until analysis.

Tissue reduced glutathione levels were assayed by the method of Ellman (1959). All tissues were weighed. Lung and kidney were homogenized in 1 ml 0.1 M pH 7.4 phosphate buffer; liver was homogenized with 4 ml of the same buffer. A 1 ml aliquot of each homogenate was added to 1 ml 4% sulfosalicyclic acid in a centrifuge tube and the mixture was vortexed and kept at 4°C. After centrifugation at 3,000 xg for 30 min, a 0.5 ml aliquot of the supernatant was removed and added to 4.5 ml DTNB reagent (0.1 mM 5,5'-dithiobis-2-nitrobenzoic acid in 0.1 M pH 8.0 phosphate buffer). Standard curves, run simultaneously with authentic glutathione, were linear from 10 to 200 µg glutathione/tube.

Covalently bound naphthalene metabolites were assayed in the fraction precipitated by sulfosalicylic acid. Unbound metabolites were removed by a series of solvent extractions with methanol and ethanol/ether (3:1) until no further radioactivity could be removed. This procedure previously has been shown to remove all of the unbound radioactivity. The precipitated macromolecular fraction was then dissolved in 1 ml 1 N NaOH, an aliquot was counted in 5 ml ACS (Amersham Searle) for 20 min in a Beckman 3150 scintillation counter and a further aliquot was taken for protein determination by the method of Lowry et al. (1951). Counts were corrected for quench by internal standardization with ^{14}C-toluene. Data are expressed as nmoles bound/mg protein.

The dose response covalent binding and glutathione depletion data are shown in Figure 2. Increasing doses of naphthalene from 25 to 200 mg/kg resulted in a moderate decrease in tissue glutathione levels in the liver and lung but not kidney. As the dose increased from 200 to 400 mg/kg there was a substantial drop in tissue

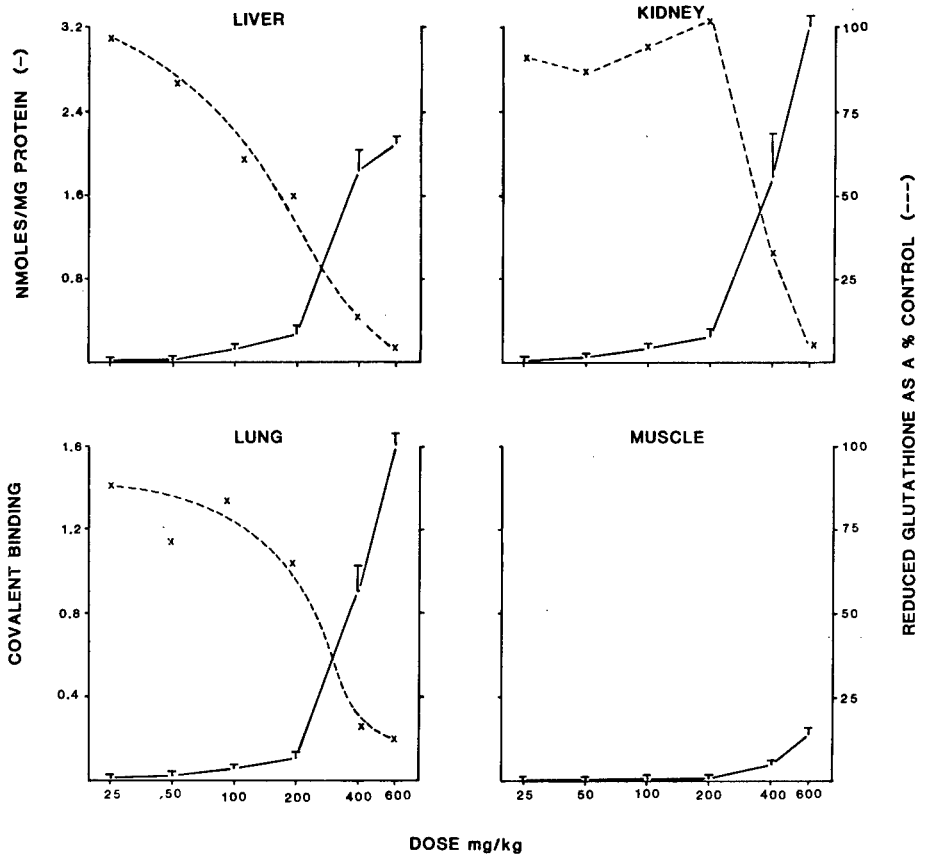


Figure 2. Dose response glutathione depletion and covalent binding 4-hours after ip administration of 1-^{14}C naphthalene. Glutathione levels are plotted as a percentage of that in tissues from reliable treated controls. Control levels ($\bar{x}$ ± S.E., n = 4) were: lung 706 ± 36 µg/g; liver 2434 ± 175 µg/g; and kidney 1420 ± 65 µg/g. Values for covalent binding are the mean ± S.E. for 3 to 4 animals.

glutathione levels in lung, liver and kidney. Pulmonary, hepatic and renal glutathione levels were less than 10% of control at the 600 mg/kg dose. While the covalent binding of reactive naphthalene metabolites to lung, liver and kidney macromolecules increased moderately as the dose of naphthalene increased from 25 to 200 mg/kg, the covalent binding levels increased markedly (more than four fold) when the dose of naphthalene doubled from 200 to 400 mg/kg. This suggests that at low doses reactive naphthalene metabolites preferentially combine with glutathione. When tissue

glutathione levels become depleted, reactive naphthalene metabolites bind covalently to tissue macromolecules. This apparent dose threshold for covalent binding which depends upon the nearly total depletion of tissue glutathione is similar to that seen with the metabolically activated hepatotoxicants acetaminophen and bromobenzene (Mitchell et al., 1975). These data, combined with the results of earlier studies (Shank et al., 1980) showing that diethymaleate pretreatment significantly increased naphthalene induced bronchiolar damage and covalent binding, suggests that the formation and covalent binding of reactive naphthalene metabolites and the depletion of glutathione are interrelated events. The data also indicate that electrophilic naphthalene metabolites which conjugate with glutathione may migrate from cells in which they are formed. This view is supported by evidence showing that pulmonary monooxygenase enzymes (which are necessary for forming electrophilic intermediates from naphthalene) are highly localized in Clara cells and type II cells while reduced glutathione appears to be evenly distributed in all lung cell types. The nearly total depletion of pulmonary glutathione which occurs after high doses of naphthalene thus indicates that electrophilic metabolites of naphthalene generated either in monooxygenase containing pulmonary cells or in the liver must reach a majority of cells of the lung. Additional support for this proposal comes from comparing the data on naphthalene with data from similar studies on 4-ipomeanol. This furan derivative undergoes metabolic activation in Clara cells to form highly reactive, cytotoxic intermediates which bind at their site of formation (Boyd, 1977). Unlike naphthalene, administration of LD_{50} doses ($\frac{1}{2}$ LD_{50} doses cause severe pulmonary damage) of 4-ipomeanol results in only a 25% depletion of pulmonary glutathione (Boyd et al., 1981). These data are consistent with the inherent chemical reactivity of furan epoxides vs the reactivity arene oxides, many of which have been prepared synthetically (Horning et al., 1980).

EFFECT OF CYTOCHROME P-450 MONOOXYGENASE INHIBITORS

Piperonyl butoxide and SKF 525A are potent inhibitors of the cytochrome P-450 monooxygenases and have been used to demonstrate a causative role of metabolic activation in the target organ damage by a number of hepatic (Mitchell, 1975), renal (Hook et al., 1979) and pulmonary toxicants (Boyd, 1980). While studies reported last year showed that pretreating mice with piperonyl butoxide blocked the pulmonary damage of a subsequently administered 300 or 500 mg/kg dose of naphthalene, they were unable to demonstrate any effect of this pretreatment on the covalent binding of reactive intermediates after treatment with 200 mg/kg ^{14}C-naphthalene. The dose response

covalent binding and glutathione depletion data showed that 200 mg/kg is below the threshold dose and at this dose, reactive metabolites of naphthalene form adducts with glutathione; comparatively little binds covalently. Thus, even though piperonyl butoxide pretreatment may have markedly inhibited the formation of these reactive metabolites, the inhibition would not be reflected in covalent binding levels but rather in the quantity of thiol adducts formed. Since the utility of covalent binding studies in monitoring the fate and formation of reactive intermediates and in establishing a causative role for such metabolites in tissue damage can be demonstrated only by showing a close parallel association between covalent binding and damage in the target tissue, studies of the effects of cytochrome P-450 monooxygenase inhibitors on naphthalene-induced pulmonary damage and glutathione depletion and on reactive metabolite binding were repeated.

Groups of 5 mice each were pretreated with piperonyl butoxide (1600 mg/kg), SKF 525A (25 mg/kg) or corn oil intraperitoneally followed 30 min later by either ^{14}C labelled (115 dpm/nmole) or unlabelled naphthalene (400 mg/kg, dissolved in corn oil such that 0.1 ml was administered ip/10 g bw). Animals receiving doses of ^{14}C labelled naphthalene were sacrificed 4 hours later for covalent binding and glutathione determination (vide infra) while animals receiving unlabelled hydrocarbon were sacrificed by pentobarbital overdose 24 hours later for histopathology. Lungs and heart were removed en block and fixed along with a 5 mm slice of the liver and one kidney in 10% buffered formalin. Tissues were paraffin embedded, sectioned at 5-6 μ and stained with hematoxylin and eosin for examination by light microscopy.

Light micrographs showing sections of terminal bronchiolar airways from mice treated with corn oil, corn oil + 400 mg/kg naphthalene, SKF 525A + 400 mg/kg naphthalene and piperonyl butoxide + 400 mg/kg naphthalene are shown in Figure 3A through 3D respectively. As shown in the 1980 annual report (Shank et al., 1980), large doses of naphthalene, administered intraperitoneally, cause extensive disruption, necrosis and exfoliation of pulmonary bronchiolar epithelial cells (Figure 3B). This bronchiolar damage was not prevented by pretreatment with 25 mg/kg SKF 525A (Figure 3C) but was decreased substantially by pretreatment with 1600 mg/kg piperonyl butoxide (Figure 3D).

The data in Figure 4 show the effects of SKF 525A or piperonyl butoxide pretreatment on glutathione depletion by naphthalene and on the covalent binding of reactive naphthalene metabolites. Consistent with previous dose response (Figure 2) and time course

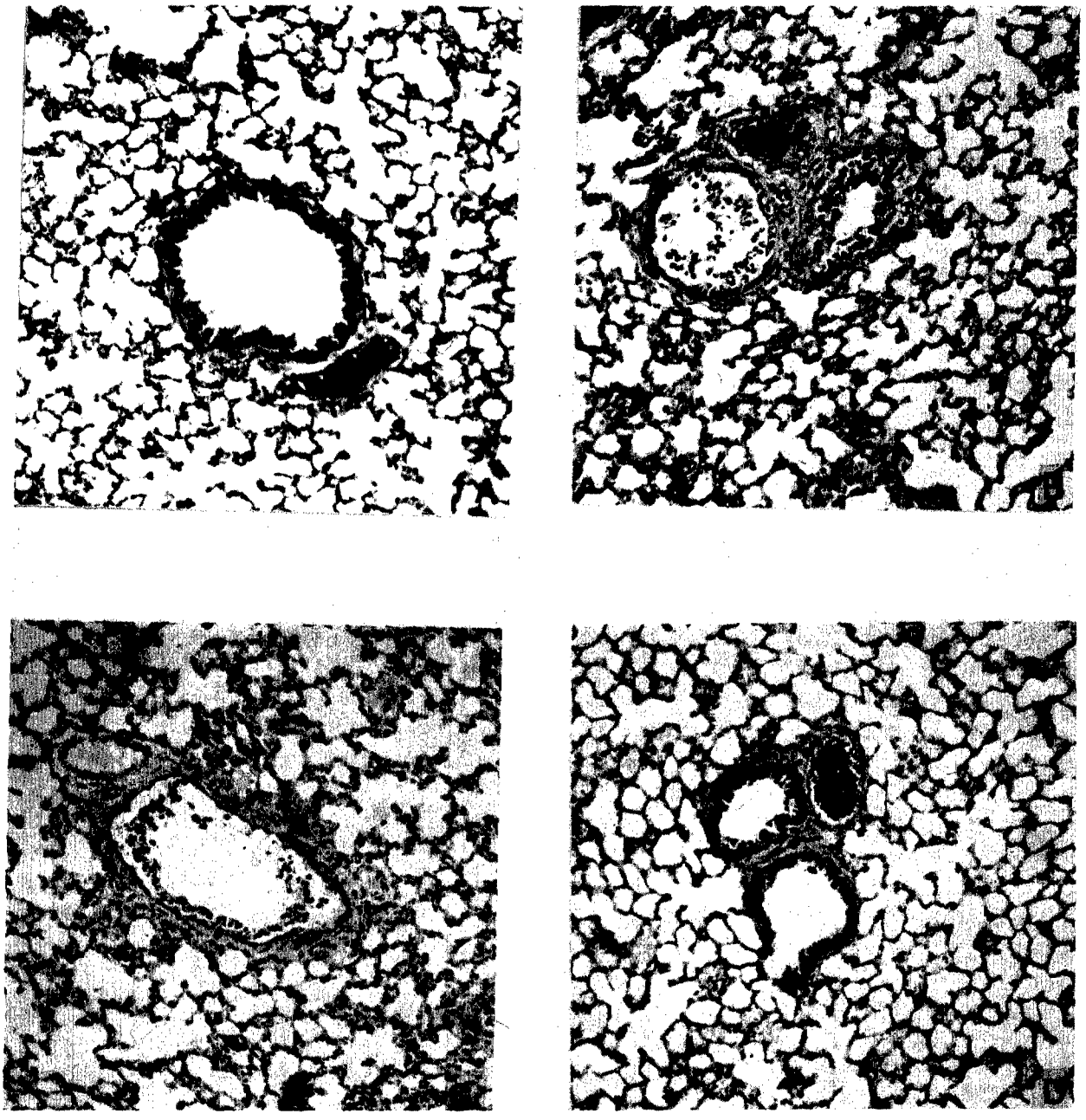


Figure 3. Light micrographs of terminal bronchiolar airways from mice treated with A) corn oil, B) corn oil plus 400 mg/kg naphthalene, C) SKF 525A plus 400 mg/kg naphthalene, D) piperonyl butoxide plus 400 mg/kg naphthalene. Tissues were embedded in paraffin, cut in 5-6μ sections and stained with hematoxylin and eosin. Original magnification x100.

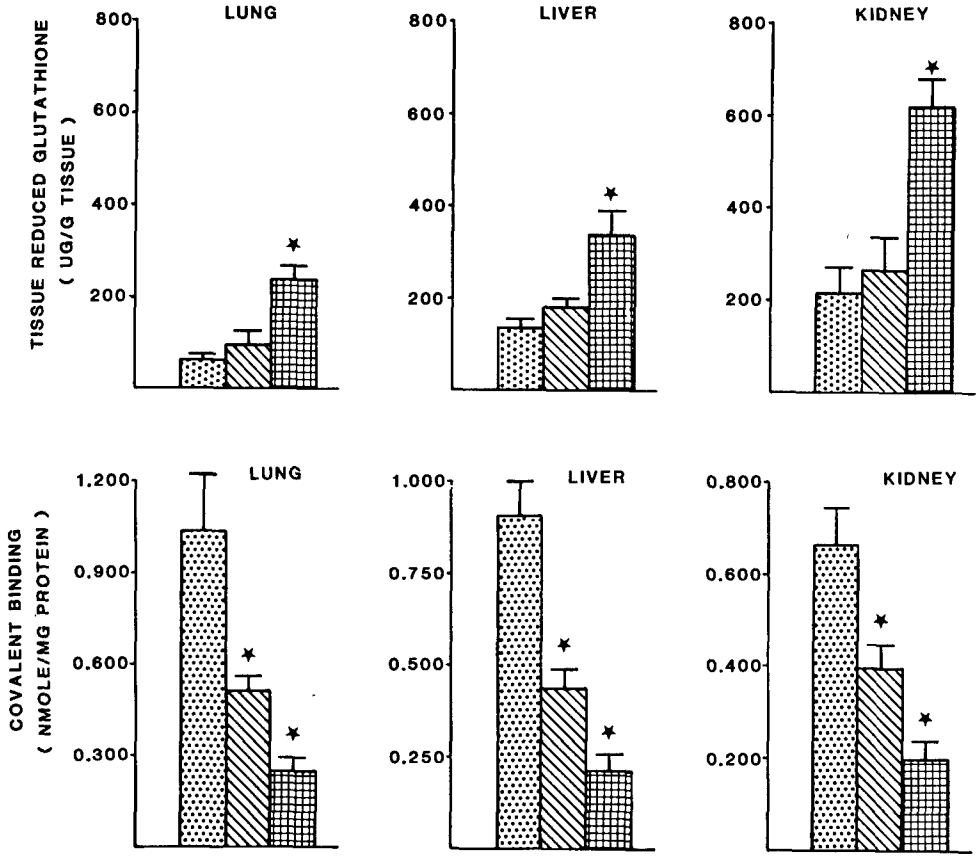


Figure 4. Effect of SKF 525A or piperonyl butoxide pretreatment on naphthalene-induced glutathione depletion and on the covalent binding of reactive naphthalene metabolites. Values are the means ± S.E. for 5 animals. Stars indicate a significant difference from vehicle plus naphthalene treated group ($p < .05$ two tailed T test).

studies (Shank et al., 1980), 400 mg/kg doses of naphthalene cause substantial glutathione depletion from tissues. (Earlier experiments have shown that glutathione levels in corn oil injected mice are approximately 700, 2400 and 1400 µg/g in tissue, lung, liver and kidney, respectively). Treatment with SKF 525A or iperonyl butoxide alone does not alter tissue glutathione levels (Mitchell et al., 1975). Pretreating mice with SKF 525A failed to block the substantial depletion of pulmonary, hepatic or renal glutathione by 400 mg/kg naphthalene. In contrast, tissue glutathione levels in mice treated with piperonyl butoxide plus naphthalene were significantly higher than those in corn oil plus naphthalene treated mice. Pretreating mice with SKF 525A or with piperonyl butoxide decreased the covalent binding in lung, liver and kidney to about 50% and 25% respectively of that in the vehicle plus

naphthalene treated group. These results support the involvement of reactive naphthalene metabolites, which at low levels are efficiently detoxified by glutathione, in the lung damage by naphthalene.

EFFECT OF PRETREATMENT WITH CYTOCHROME P-450 MONOOXYGENASE ENZYME INDUCERS

In many instances, induction of the cytochrome P-450 monooxygenase enzymes has provided evidence for the causative role of reactive metabolites in tissue damage. Moreover, in some cases xenobiotic metabolizing enzyme induction has been important in demonstrating the role of extrahepatic metabolism in chemical-induced damage to organs like lung or kidney. For example, the hepatic and renal damage induced by $CHCl_3$ in mice appears to be due to the generation of reactive $CHCl_3$ metabolite(s) (Ilett et al., 1973). Pretreatment of mice with 3-methylcholanthrene or polychlorinated biphenyls increased the hepatotoxicity and decreased the renal toxicity of $CHCl_3$ thus suggesting that the nephrotoxic metabolite of $CHCl_3$ is generated in the kidney and not the liver (Kluwe et al., 1978).

In an attempt to provide further evidence for the role of reactive metabolites generated in situ in the lung in naphthalene-induced bronchiolar damage, the effects of phenobarbital or 3-methylcholanthrene pretreatment on naphthalene induced tissue damage, covalent binding and glutathione depletion have been studied.

Male Swiss Webster mice (22-32 g, Simonsen Breeding Labs) were randomly divided into groups of 4-8 animals each. All dose solutions were prepared such that 0.1 ml was administred intraperitoneally per 10 g bw. Control animals received injections of vehicle (saline, corn oil). Phenobarbital (PB)-treated animals were given twice daily injections of 50 mg/kg (in saline) for 5 days with the last dose being administered 24 hours before naphthalene. 3-Methylcholanthrene (3MC), dissolved in corn oil, was administered at 80 mg/kg, 72 and 48 hours prior to naphthalene. Doses of unlabelled or ^{14}C-labelled naphthalene (Amersham Searle, >99.5% radiochemical purity by HPLC) were given at 50, 100, 200 or 400 mg/kg in corn oil.

Four mice from each of the pretreatment groups (control, PB, 3MC) were sacrificed by cervical dislocation and hepatic microsomes were prepared from individual livers by differential ultracentrifugation (Mazel, 1971). Microsomal cytochrome P-450 was

determined in washed microsomes by the method of Omura and Sato (1964) on a Cary 210 spectrophotometer. The data in Table 1 show that phenobarbital pretreatment produced the characteristic increase in liver weight, microsomal yield and cytochrome P-450 level. 3-Methylcholanthrene pretreatment did not stimulate an increase in liver weight or microsomal yield and produced only a marginal increase in cytochrome P-450/P-448 which was different from control only at the P<.10 level of significance.

TABLE 1. EFFECT OF PHENOBARBITAL OR 3-METHYLCHOLANTHRENE TREATMENT ON LIVER WEIGHT, YIELD OF HEPATIC MICROSOMES AND MICROSOMAL CYTOCHROME P-450[a]

Treatment	Liver Wt(g)	Microsomal Yield mg/g	Cytochrome P-450 nmoles/mg prot.
Saline/corn oil	1.75±0.04	15.71±0.94	1.22±0.08
Phenobarbital	2.28±0.19[b] (130)	21.62±2.08[b] (138)	2.55±0.14[b] (209)
3-Methyl-cholanthrene	1.73±0.05 (99)	15.02±1.01 (96)	1.55±0.12[c] (127)

[a] Values are the means ± S.E. for livers 4 mice. Numbers in parentheses indicate values expressed as a percentage of vehicle treated control.

[b] Indicates a significant difference from vehicle treated control $P < .05$ two tailed T test.

[c] Indicates a significant difference from vehicle treated control $P < 0.1$ two tailed T test.

Twenty-four hours after treatment with unlabelled naphthalene, lung, liver and kidney were removed from mice for examination by light microscopy. The data in Table 2 summarize the effects of pretreatment with phenobarbital or 3-methylcholanthrene on % mortality and the site of tissue necrosis after naphthalene. Necrotic cells were not evident in lung, liver or kidney in any of the pretreatment groups treated with either 50 or 100 mg/kg naphthalene. Lungs from mice pretreated with vehicle, PB or 3MC and given 200 mg/kg showed slight swelling of terminal bronchiolar cells (Figure 5). No differences were apparent either in the size of the airways involved or the relative numbers of bronchiolar cells affected when the different pretreatment groups receiving 200 mg/kg were compared. In all of the pretreatment groups receiving 400 mg/kg there was extensive necrosis and exfoliation of

TABLE 2. EFFECT OF PHENOBARBITAL OR 3-METHYLCHOLANTHRENE PRETREATMENT ON MORTALITY AND TISSUE NECROSIS 24 HOURS AFTER NAPHTHALENE[a]

Pretreatment	Dose of Naphthalene (mg/kg)	Mortality %	Site of Tissue Necrosis[b] Lung	Liver	Kidney
Saline/corn oil	0	0	0	0	0
	50	0	0	0	0
	100	0	0	0	0
	200	0	+	0	0
	400	20	++	0	++[c]
Phenobarbital	50	0	0	0	0
	100	0	0	0	0
	200	0	+	0	0
	400	40	++	+[d]	0
3-Methylcholanthrene	50	0	0	0	0
	100	0	0	0	0
	200	0	+	0	0
	400	20	++	+[d]	0

[a] Groups of 5 mice each were pretreated with vehicle or inducer as described in the text and were then treated with naphthalene at the specified dose. Twenty-four hours later, animals were sacrificed by pentobarbital overdose and lungs, livers and kidneys were removed, placed in 10% buffered formalin, embedded in paraffin and stained with hematoxylin and eosin.

[b] Tissue lesions were scored as either slight (+) or extensive (++). In lungs graded + only a few cells of the most terminal bronchiolar airways were necrotic and had sloughed into the lumen. In extensively damaged lungs, there was exfoliation and necrosis of most epithelial cells in a majority of the bronchiolar airways.

[c] Extensive proximal tubular necrosis occurred in 2 of the four control animals treated with 400 mg/kg naphthalene.

[d] Hepatic necrosis occurred in 2/3 of PB + naphthalene 400 mg/kg treated animals and in 2/4 of 3MC + naphthalene 400 mg/kg treated animals.

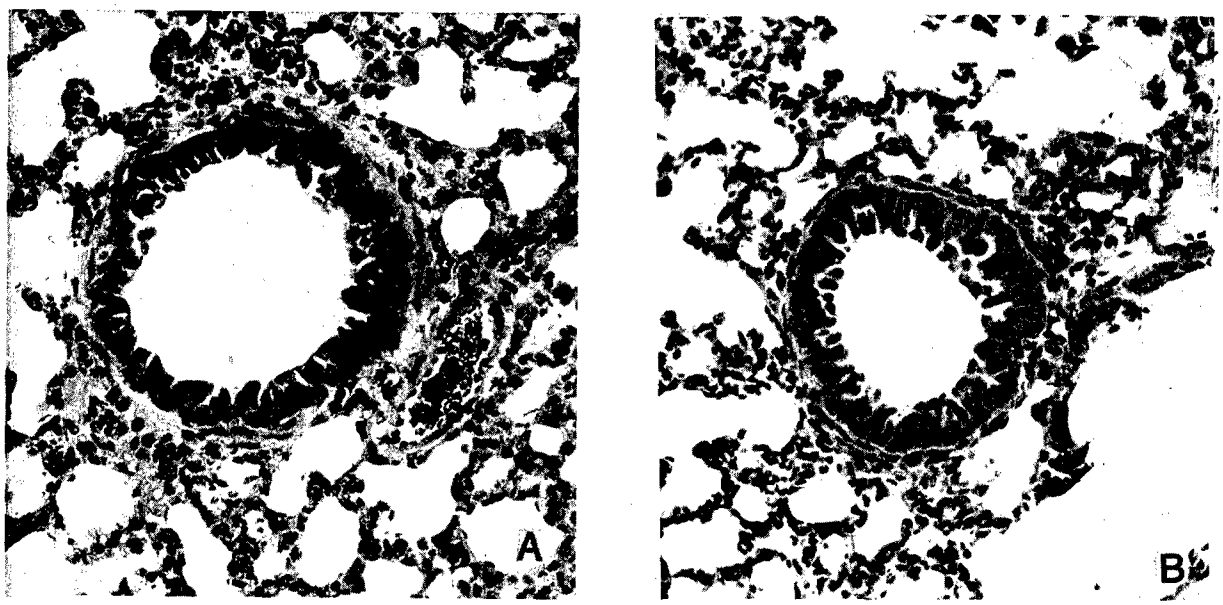


Figure 5. Light micrographs of terminal bronchiolar airways from vehicle (A), phenobarbital (B) or 3-methylcholanthrene (C) pretreated mice treated intraperitoneally with 200 mg/kg naphthalene 24 hours earlier. H + E stain. Original magnification x 200.

bronchiolar epithelial cells. Again no differences between the pretreatment groups were discernible.

In two of the four surviving mice pretreated with vehicle and given 400 mg/kg naphthalene there was extensive renal tubular necrosis (Figure 6). This has not been observed previously in kidneys of non-pretreated animals receiving a 400 mg/kg dose (a total of 12 have been examined) of naphthalene. The underlying basis for this result is not clear. All animals were approximately the same age and were handled in the same manner as those examined previously. No necrosis was observed in kidneys from PB or 3MC pretreated animals given 400 mg/kg naphthalene.

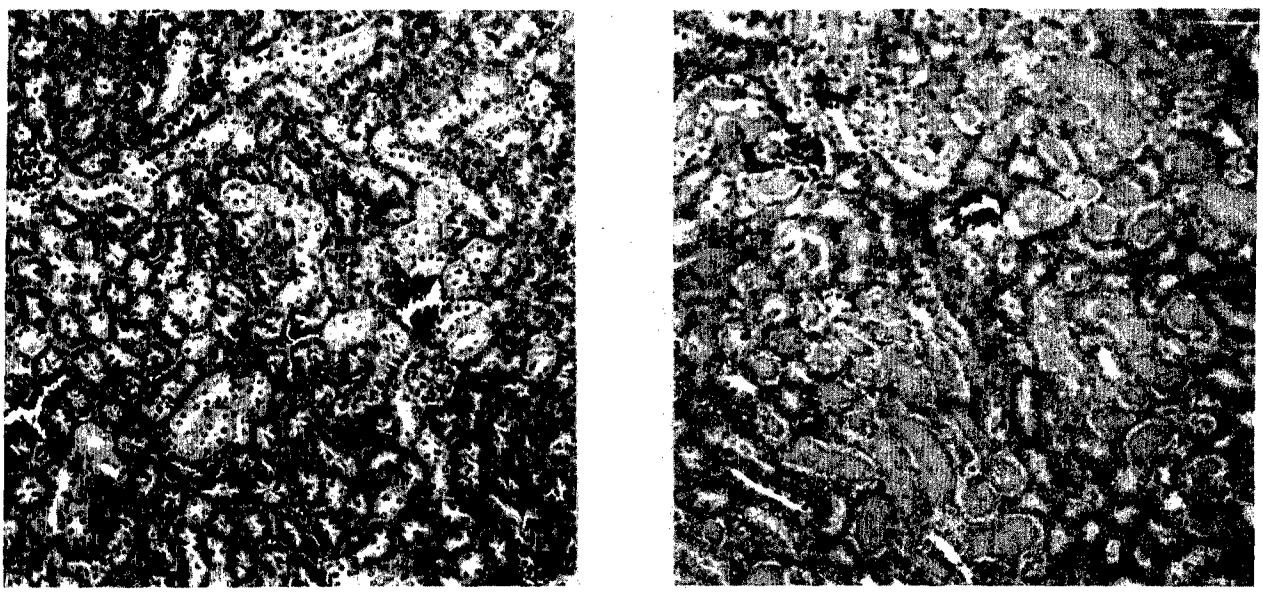
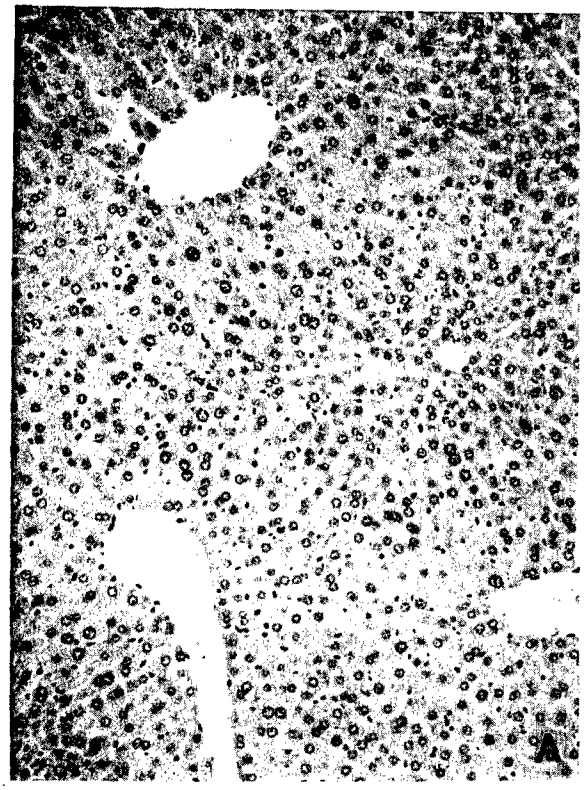
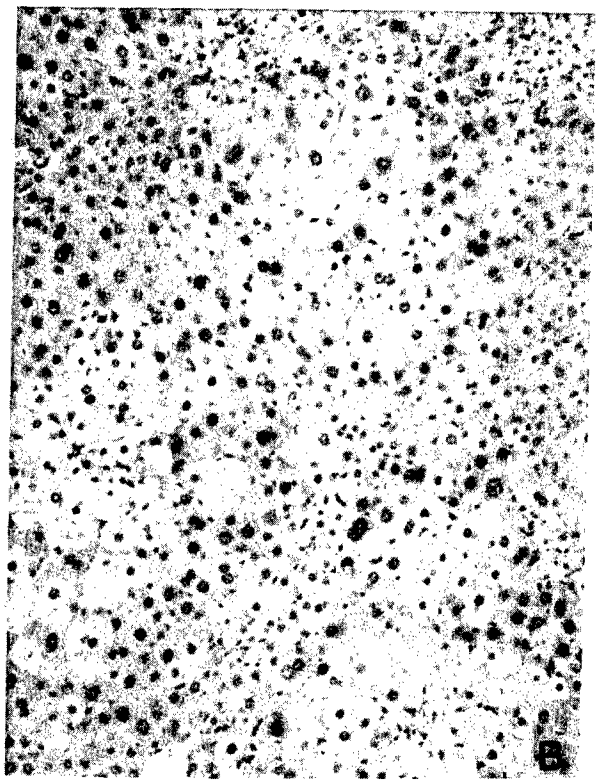


Figure 6. Light micrographs of kidneys from vehicle treated (control) (A) and naphthalene treated (500 mg/kg i.p.) mice (B). H + E stain, original magnification x 100.

In two of the three surviving PB plus naphthalene 400 mg/kg and 2 out of the 4 of the 3MC plus naphthalene treated mice there were focal areas of hepatic necrosis not noted in vehicle + naphthalene treated animals (Figure 7).

Twenty mice from each pretreatment group were given doses of ^{14}C-naphthalene (50, 100, 200 or 400 mg/kg) intraperitoneally and the animals were sacrificed 4 hours later for the determination of





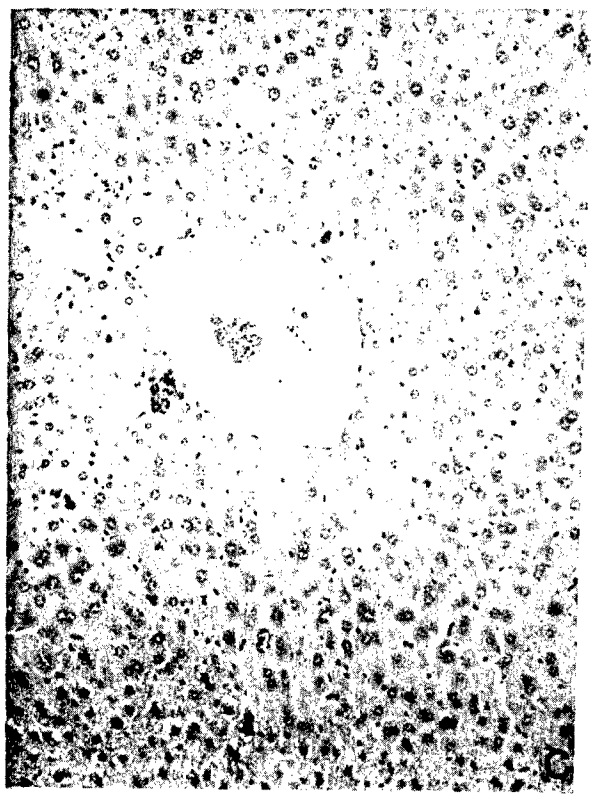


Figure 7. Light micrographs of livers from vehicle (A), phenobarbital (B) or 3-methylcholanthrene (C) pretreated mice given 400 mg/kg naphthalene intraperitoneally. H + E stain, original magnification x 100.

reduced glutathione levels and covalently bound reactive metabolites as described previously. The data in Figure 8 compare the dose response for glutathione depletion and covalent binding in vehicle, PB and 3MC pretreated mice. Glutathione levels are plotted as a % of those levels in mice pretreated with vehicle, PB or 3MC and given corn oil. In lung and liver of vehicle pretreated mice, naphthalene produced a dose dependent depletion of glutathione. Glutathione levels in the kidney remain near control levels at the 50, 100 and 200 mg/kg doses of naphthalene and only at the 400 mg/kg dose is substantial glutathione depletion from the kidney observed. These results are similar to those obtained in a previous experiment (Figure 2). While at several doses of naphthalene there are significant elevations of tissue glutathione levels in PB pretreated mice and depressions of glutathione concentrations in 3MC pretreated animals compared to vehicle controls, pretreatment with these xenobiotic metabolizing enzyme inducers does not appear to cause a significant shift in the dose response curve.

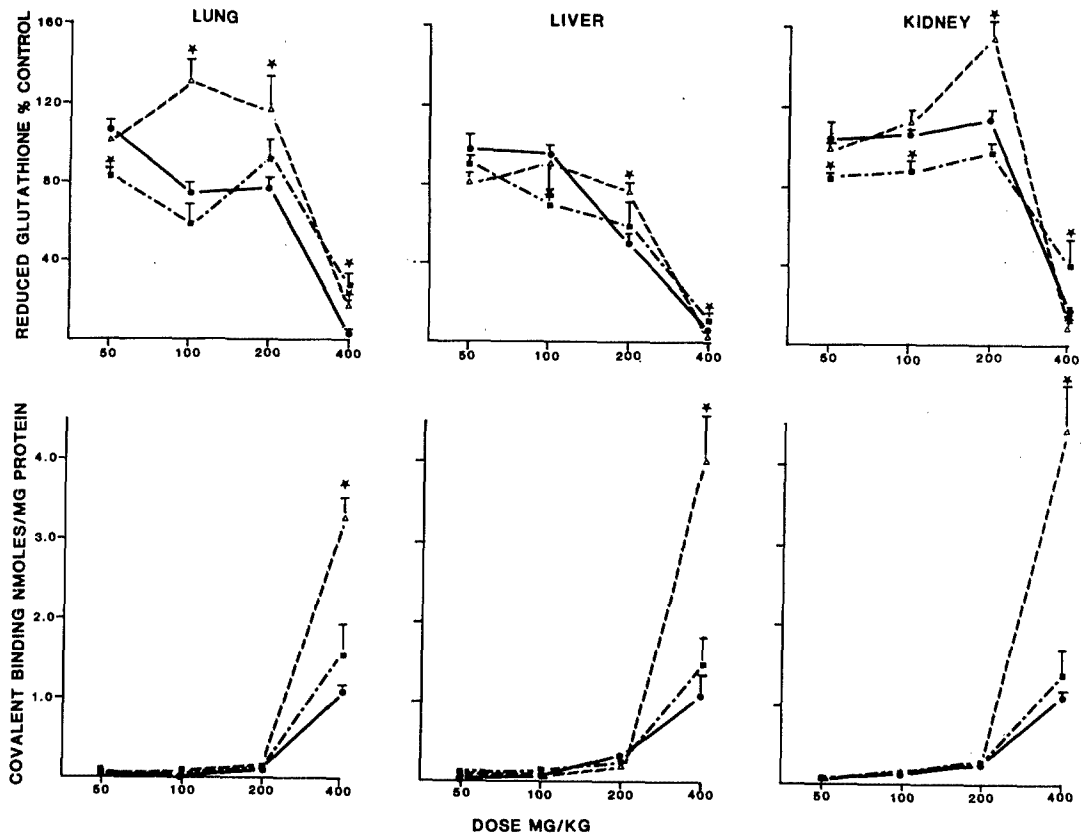


Figure 8. Dose response for naphthalene induced glutathione depletion and covalent binding in control (● —— ●), phenobarbital (▲- - -▲) and 3-methylcholanthrene (■· —·■) pretreated animals. All values are the means ± S.E. for 5 animals. Glutathione values are plotted as a percentage of their respective controls. Stars indicate a significant difference from vehicle treated animals, $p < .05$, two tailed t test).

Phenobarbital pretreatment did not alter the levels of covalently bound naphthalene metabolites in lung, liver or kidney in animals treated with 50, 100 or 200 mg/kg ^{14}C-naphthalene when compared to vehicle pretreated controls. However, covalent binding in PB induced animals given 400 mg/kg ^{14}C-naphthalene was three to four fold higher than in control animals given 400 mg/kg ^{14}C-naphthalene. No change in the relative ratios of covalent binding in lung to liver to kidney to muscle was noted in PB vs control animals suggesting that there may be a common site for the formation of reactive metabolites which become bound to tissue macromolecules. Likewise 3MC pretreatment did not alter the levels of covalent binding in any of the tissues studied at the 50, 100 or 200 mg/kg dose of naphthalene. Although covalent binding in lung and liver in 3MC pretreated animals appeared greater than in control at the 400 mg/kg dose of naphthalene (146 and 138% of control in lung and liver respectively), the increase was not statistically significant. This may be due to the relatively small induction of cytochrome P-450 by 3MC in this experiment.

This study did not provide any further evidence on the possible role of reactive metabolites in pulmonary damage by naphthalene. Pretreatment with either inducer did not alter covalent binding levels at the 200 mg/kg dose of naphthalene nor did it produce any observable change in the bronchiolar damage by naphthalene at this dose. Although covalent binding was much higher in lungs of PB pretreated animals compared to controls at 400 mg/kg naphthalene, damage to bronchiolar airways of vehicle pretreated animals given 400 mg/kg naphthalene was so extensive that it is doubtful that any additional tissue damage could be observed.

The effects of PB or 3MC pretreatment on the tissue damage and on covalent binding and glutathione depletion caused by an agent like naphthalene cannot be predicted a priori. Both PB and 3MC increase the activities of not only the monooxygenases but also potential detoxication enzymes such as epoxide hydrolase and glucuronyl transferase (Neal, 1980). Induction of potential detoxifying enzyme systems by PB and 3MC could explain why neither agent altered the dose threshold for covalent binding and glutathione depletion. It appears that the marked increase in reactive metabolite binding occurs only after all detoxifying pathways have been saturated.

Origin of Covalently Bound Bronchiolar Cytotoxic Metabolites

Previous studies described in this and last years' annual report (Shank et al., 1980) have indicated that hepatic metabolism of

naphthalene may play a role in forming derivatives which are either directly cytotoxic in the lung or which can be further metabolized by pulmonary enzymes to a cytotoxic intermediate(s). The various possibilities are discussed in the Introduction to this report and are outlined in Figure 1. Two experimental approaches have been taken in an attempt to determine the role of hepatic metabolism in naphthalene induced lung damage: 1) selectively destroy pulmonary cytochrome P-450 monooxygenases with p-xylene and 2) compare the tissue damage, covalent binding and glutathione depletion after administration of naphthalene by intraperitoneal, oral and inhalation routes.

EFFECT OF P-XYLENE PRETREATMENT ON NAPHTHALENE INDUCED PULMONARY DAMAGE AND ON THE COVALENT BINDING AND GLUTATHIONE DEPLETION BY NAPHTHALENE METABOLITES

Administration of high doses of p-xylene (1 g/kg) to rats or rabbits has been shown previously to selectively damage cytochrome P-450 monooxygenases in the lung (Patel et al., 1978, 1979). These studies have been repeated in mice with similar results (Shank et al., 1980). The inactivation of pulmonary P-450 by p-xylene appears to depend upon its metabolism to p-tolualdehyde which can be detoxified by aldehyde dehydrogenses in the liver but not the lung. Thus, it seemed that p-xylene pretreatment might afford the opportunity to determine whether lung monooxygenase enzymes play an obligatory role in forming toxic and/or covalently bound metabolites from naphthalene.

Male Swiss Webster mice (28-40 g), from Simonsen Breeding Labs, were divided into groups of 4 each and were treated as outlined in Table 3. Naphthalene and p-xylene were dissolved in corn oil (0.1 ml/10 g bw) and were administered intraperitoneally. Naphthalene was administered 24 hours after the pretreatment and all animals were sacrificed by pentobarbital overdose 24 hours after naphthalene. Lung, liver and kidney were removed for histopathology by methods described previously.

Light microscopic examination of lungs from mice sacrificed 24 hours after the administration of p-xylene showed only slight differences from lungs of vehicle pretreated animals. In p-xylene treated mice there were occasional swollen epithelial cells in a few of the bronchiolar airways but no apparent necrosis was observed thus confirming previous reports (Harper et al., 1977) (Figure 9). Lungs of mice examined 48 hours after p-xylene were indistinguishible from vehicle control. There was moderate to extensive damage to cells of the bronchiolar epithelium in lungs of

TABLE 3. EFFECT OF P-XYLENE PRETREATMENT ON NAPHTHALENE-INDUCED PULMONARY DAMAGE

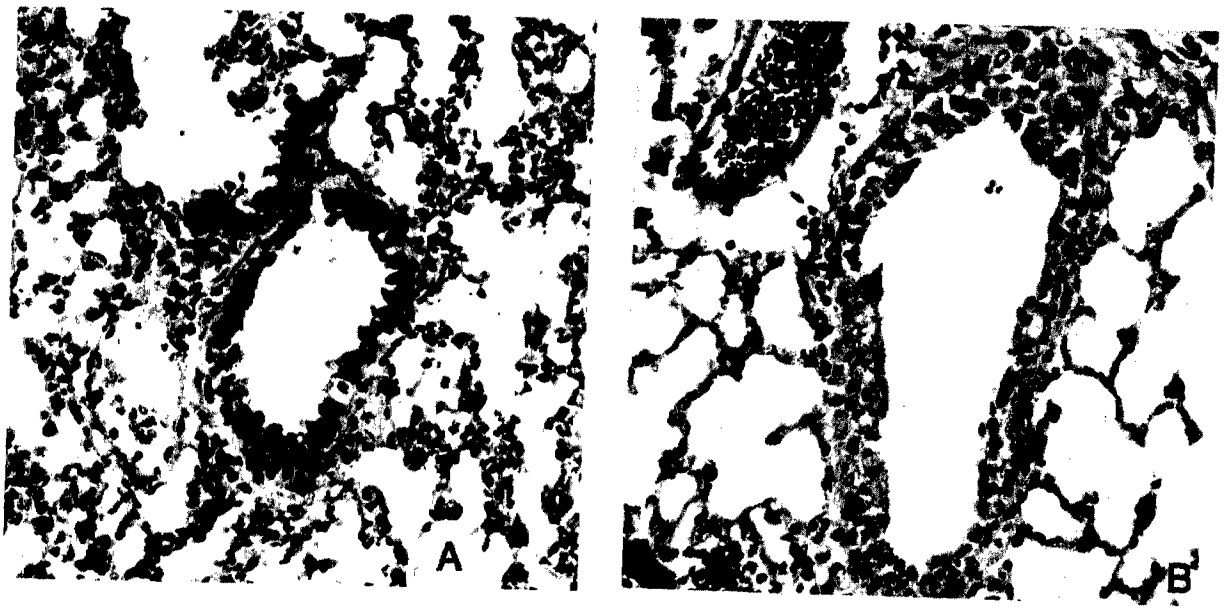
Pretreatment	Dose of Naphthalene	% Mortality	Lung Damage
Corn oil	corn oil	0	no
Corn oil	100	0	
Corn oil	200	0	yes
Corn oil	400	75	yes
P-xylene[a]	0	0	no[c]
P-xylene[b]	0	0	no
P-xylene	100	0	no
P-xylene	200	0	no
P-xylene	400	75	no

[a] Animals were sacrificed 24 hours after p-xylene treatment.
[b] Animals were sacrificed 48 hours after p-xylene treatment.
[c] There were occasional swollen epithelial cells in a few of the terminal bronchiolar airways.

mice pretreated with corn oil followed by 200 or 400 mg/kg naphthalene which was completely blocked by p-xylene pretreatment (Figure 9, Table 3). The data from this experiment indicated that pulmonary damage by naphthalene requires metabolism by monooxygenase enzymes in the lung.

A further experiment was conducted to determine whether p-xylene pretreatment would a) block glutathione depletion in the lung by naphthalene and b) decrease pulmonary covalent binding of naphthalene metabolites. Male Swiss Webster mice (25-30 g) were divided into groups of either 5 (glutathione and covalent binding studies) or 9 (cytochrome P-450 studies) each. Groups of animals for the glutathione and covalent binding studies were given either corn oil or p-xylene (1 g/kg) intraperitoneally followed 16 hours later by corn oil (glutathione control) or ^{14}C-naphthalene (400 mg/kg, 70 dpm/nmole). Animals were sacrificed four hours later for glutathione and covalent binding determinations as described earlier. The treatment protocol is outlined at the bottom of Figure 10. Four groups of 9 mice each were given ip injections of either corn oil or p-xylene (1 g/kg) and the animals were sacrificed 16 hours later for microsomal cytochrome P-450 determinations in lung and liver.

The data in Figure 10 show that p-xylene treatment substantially decreased pulmonary cytochrome P-450 levels without affecting



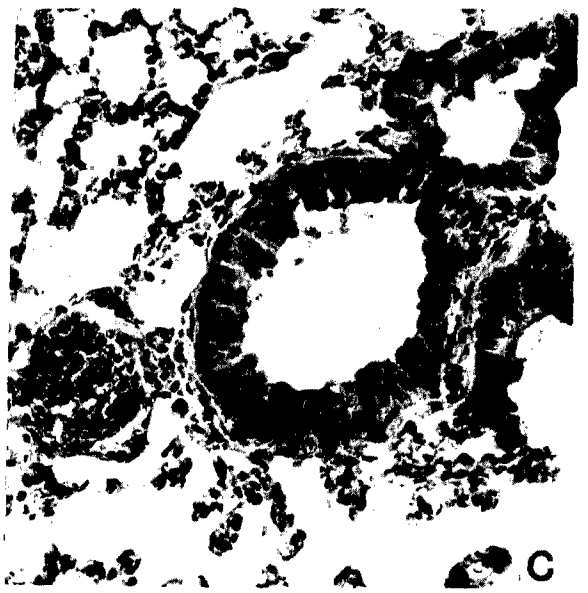


Figure 9. Light micrographs of bronchiolar airways of mice A) treated with 1 g/kg p-xylene, B) corn oil plus 400 mg/kg naphthalene, C) p-xylene 1 g/kg + 400 mg/kg naphthalene. H + E stain, original magnification x 200.

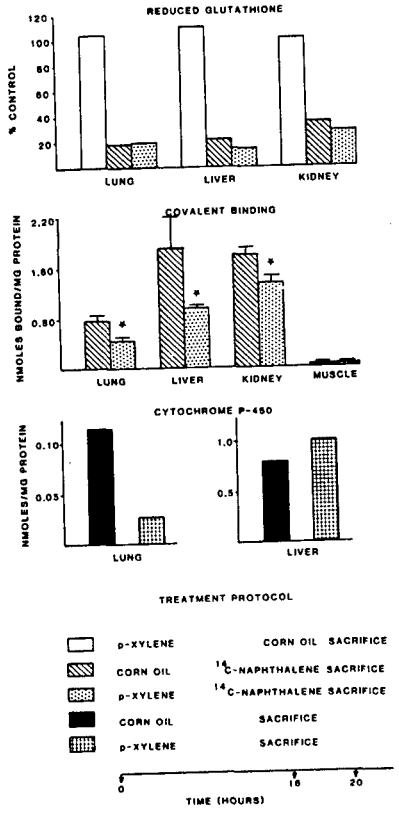


Figure 10. Effect of p-xylene pretreatment on naphthalene induced glutathione depletion, the covalent binding of reactive naphthalene metabolites and on pulmonary and hepatic cytochrome P450 levels. Glutathione levels are expressed as a percent of those in corn oil treated animals. Control glutathione levels were 653 ± 25, 2165 ± 259, and 1372 ± 16 µg/g ($\bar{X}$ + S.E., n = 5) for lung, liver and kidney respectively. Covalently bound naphthalene metabolites are expressed as $\bar{x}$ ± S.E. for 5 animals. * indicates a significant difference from corn oil pretreated animals, $p < .05$, two tailed T test. Cytochrome P450 determinations were conducted on microsomes prepared from pooled tissues of 9 animals.

hepatic levels, a result consistent with those of earlier experiments. Treatment with p-xylene alone had no effect on reduced glutathione levels in lung, liver or kidney. As seen in previous studies, pulmonary, hepatic and renal glutathione levels were decreased to 20-35% of control levels in animals treated with 400 mg/kg naphthalene. Pretreatment with p-xylene had no effect on the marked naphthalene-induced glutathione depletion but it did significantly decrease the covalent binding of reactive metabolites

from naphthalene in lung, liver and kidney. This decrease was not selective and occurred in liver and kidney as well as lung. It is possible that sufficient p-xylene remains in the liver 16 hours after treatment to competitively inhibit hepatic monooxygenase enzymes thereby decreasing the rate at which naphthalene is metabolically activated. Future studies will use lower doses of p-xylene in an attempt to produce a selective effect in the lung.

EXPOSURE BY INHALATION

Another approach to determining the relative roles of lung and liver in the formation of reactive intermediates and in the lung damage by naphthalene is to study the effects of route of administration on these parameters. Intraperitoneal administration (the route used for these studies thus far) generally results in a large percentage of total toxicant passing through the liver before reaching other organs (Lukas et al, 1971). Exposure of animals to naphthalene by inhalation would deliver the toxicant directly to the target organ. While inhalation exposure would not preclude the possibility of naphthalene undergoing uptake, distribution to and metabolism by the liver, exposure by this route might be expected to alter the relative contribution of the liver and lungs in the overall disposition and metabolism of the compound. In addition, exposure by inhalation is one of the major routes of human exposure.

Before expending the time and money to construct a flow-through exposure system, preliminary static exposure studies have been conducted to determine whether naphthalene given by inhalation produces lung lesions in the mouse similar to those observed when naphthalene is administered intraperitoneally. Three hour exposures were carried out in an 11 ℓ vacuum desiccator fitted with a truebore stirrer and a large teflon paddle. Varying amounts of naphthalene were placed beneath the porcelain plate in the bottom of the desiccator and the chamber was immersed in warm water for 1 hour to volatilize the compound. Mice were placed in the desiccator 2 hours after removing the chamber from the water. Air concentrations of naphthalene were monitored at 15 to 30 minute intervals both before and during the exposure. Naphthalene concentrations were quantitated by removing a 10 ml air sample from the chamber with a gas tight syringe, bubbling the sample into 1 ml hexane in a quartz UV cuvette and scanning the sample from 300 to 240 nm. Air samples taken in this manner yield UV scans which are identical (absorbance maxima at 286, 284, 275.5, 266 and 258 nm) to authentic naphthalene. Replicate determinations of chamber air concentrations performed at 5 min intervals revealed little intersampling variation. Because this sampling method could lead to an

underestimate of chamber air concentrations, quantitation will be checked by a gas chromatographic procedure in upcoming nose-only exposures.

Groups of 5 male Swiss Webster mice (28-37 g) were exposed to naphthalene by inhalation at concentrations of 0 (sham exposed control), 368 mg/m^3 (70 ppm), 261 mg/m^3 (50 ppm), 122 mg/m^3 (23 ppm) and 44 mg/m^3 (8 ppm). At the end of the exposure, animals were removed from the chamber. Twenty four hours later, the animals were sacrificed by pentobarbital overdose and lungs were fixed, embedded, cut and stained as described previously. Light micrographs of terminal bronchiolar airways from control and naphthalene exposed mice are shown in Figure 11. Bronchiolar epithelial cells in sham exposed animals appeared slightly disrupted and this may be a result of the relatively lengthy static exposure. Even at the lowest concentration of naphthalene studied (44 mg/m^3), necrosis of bronchiolar epithelial cells was observed. At this and higher concentrations of naphthalene there was extensive exfoliation of epithelial cells into the bronchiolar lumen. At 24 hours, there were no deaths at any of the concentrations of naphthalene studied. Thus, although these studies are preliminary, they demonstrate that lung damage produced by naphthalene given ip can be elicited by inhalation exposure even at very low concentrations. In future studies animals will be exposed in a nose-only exposure system. Pharmacokinetic studies on naphthalene disposition and metabolism after administration by intraperitoneal and inhalation routes may provide further evidence concerning the relative roles of lung and liver in the formation of covalently bound, glutathione depleting metabolites of naphthalene that may be important in the lung damage.

COMPARATIVE METABOLISM AND TOXICOLOGIC STUDIES

Species Differences in Naphthalene-Induced Tissue Damage

One of the long-term goals of the research in the Part II, Subprogram in Comparative Metabolism and Biochemistry is to identify the critical molecular events in naphthalene induced pulmonary damage. In order to identify such events comparative metabolic studies will be carried out in target vs non target tissues and in sensitive vs non sensitive species. Initial studies have now been completed in species other than the mouse to determine whether i.p. administration of naphthalene causes tissue damage.

Male Golden Syrian hamsters (65-85 g) were divided randomly into groups of 5 each and were administered doses of naphthalene

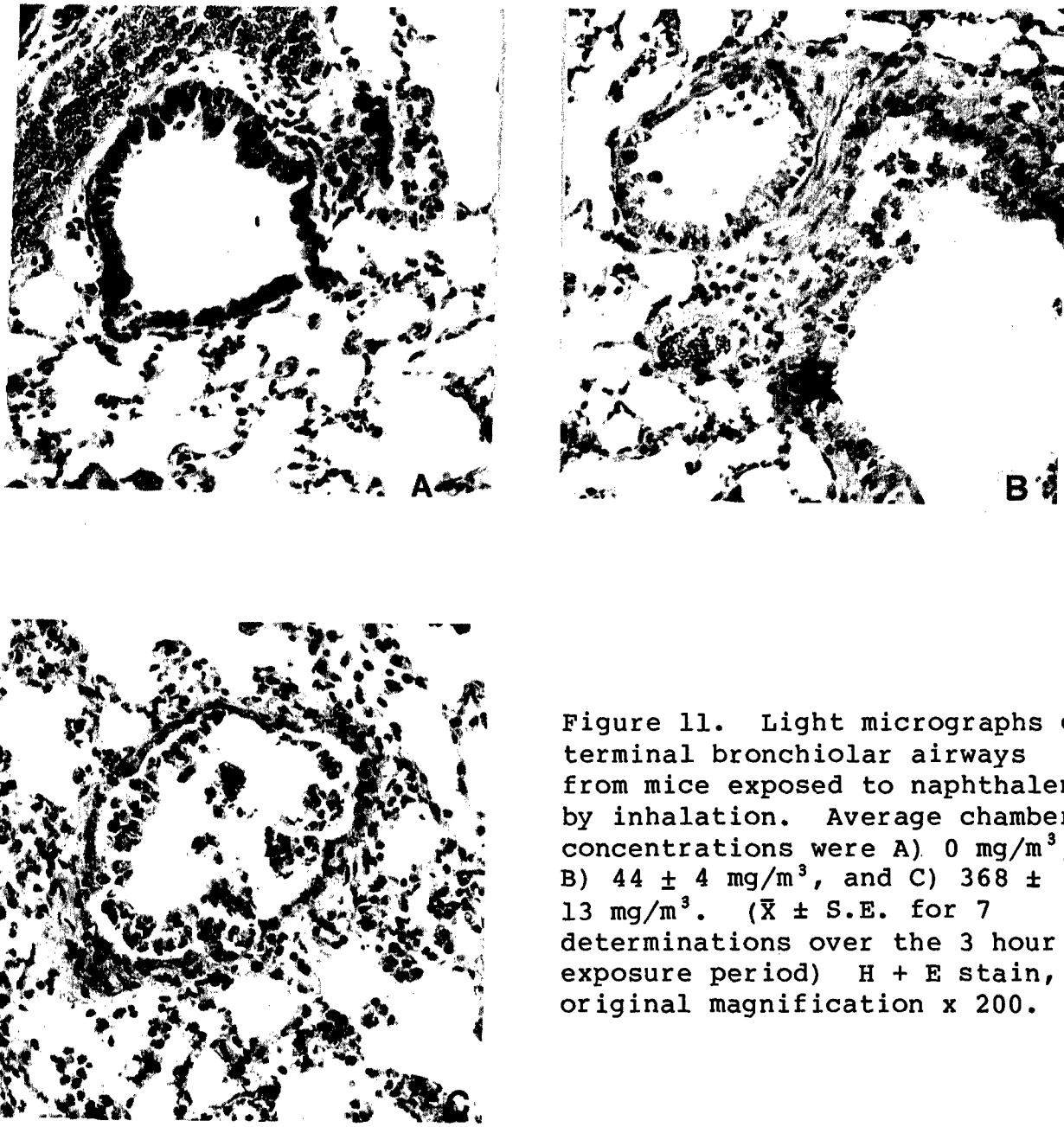


Figure 11. Light micrographs of terminal bronchiolar airways from mice exposed to naphthalene by inhalation. Average chamber concentrations were A) 0 mg/m^3, B) 44 ± 4 mg/m^3, and C) 368 ± 13 mg/m^3. ($\bar{X}$ ± S.E. for 7 determinations over the 3 hour exposure period) H + E stain, original magnification x 200.

intraperitoneally ranging from 25 to 800 mg/kg. Twenty-four hours later surviving animals were sacrificed by pentobarbital overdose and lungs and heart were removed en block. The trachea was cannulated and lungs were perfused with 10% buffered formalin for 72 hours at 30 cm pressure. Three to 6 mm sections of liver were cut with a razor blade and fixed along with kidney. Initially all tissues were embedded in paraffin, sectioned at 5-6 μ and stained with hematoxylin and eosin. A more detailed examination of the lungs required that this tissue be embedded in methacrylate, sectioned at 1μ and stained with toluidine blue.

Table 4 presents the data on percent mortality and site of tissue necrosis in hamsters after varying doses of naphthalene. These data indicate that the acute toxicity of naphthalene in the hamster is less than in the mouse (LD_{50}, 380 mg/kg, ip). In a subsequent study, which is currently not completed, using higher doses and different sacrifice times, the 24 hour mortality by naphthalene in hamsters was 20% at 1200 mg/kg and 60% at 1600 mg/kg. In the time course study, of the 5 hamsters given ip doses of naphthalene at 800 mg/kg, all survived 72 hours.

TABLE 4. MORTALITY AND SITE OF TISSUE NECROSIS 24 HOURS AFTER IP ADMINISTRATION OF NAPHTHALENE IN THE HAMSTER

Dose of Naphthalene mg/kg	Mortality %	Site of Tissue Necrosis		
		Lung	Liver	Kidney
Vehicle (corn oil)	0	0	0	0
25	0	0	0	0
50	0	0	0	0
100	0	0	0	0
200	0	0	0	0
400	0	0	0	0
800	20	+[a]	+[b]	0

[a] There was marked swelling and vacuolation of cells of the terminal bronchiolar airways.

[b] In hamsters treated with 400 mg/kg naphthalene, small numbers of centrilobular hepatic cells were necrotic. In animals treated with 800 mg/kg naphthalene, necrosis was observed in large numbers of centrilobular cells.

As indicated in Table 4 centrilobular necrosis was observed in the 400 mg/kg treatment group which was considerably more extensive in the 800 mg/kg treatment groups. Damage to bronchiolar epithelial

cells in the hamster was noted only in the highest dose of naphthalene tested (800 mg/kg). Cells of the bronchiolar epithelium were highly swollen and vacuolated but did not appear to be sloughed into the lumen of the bronchiole as they are in the mouse (Figure 12).

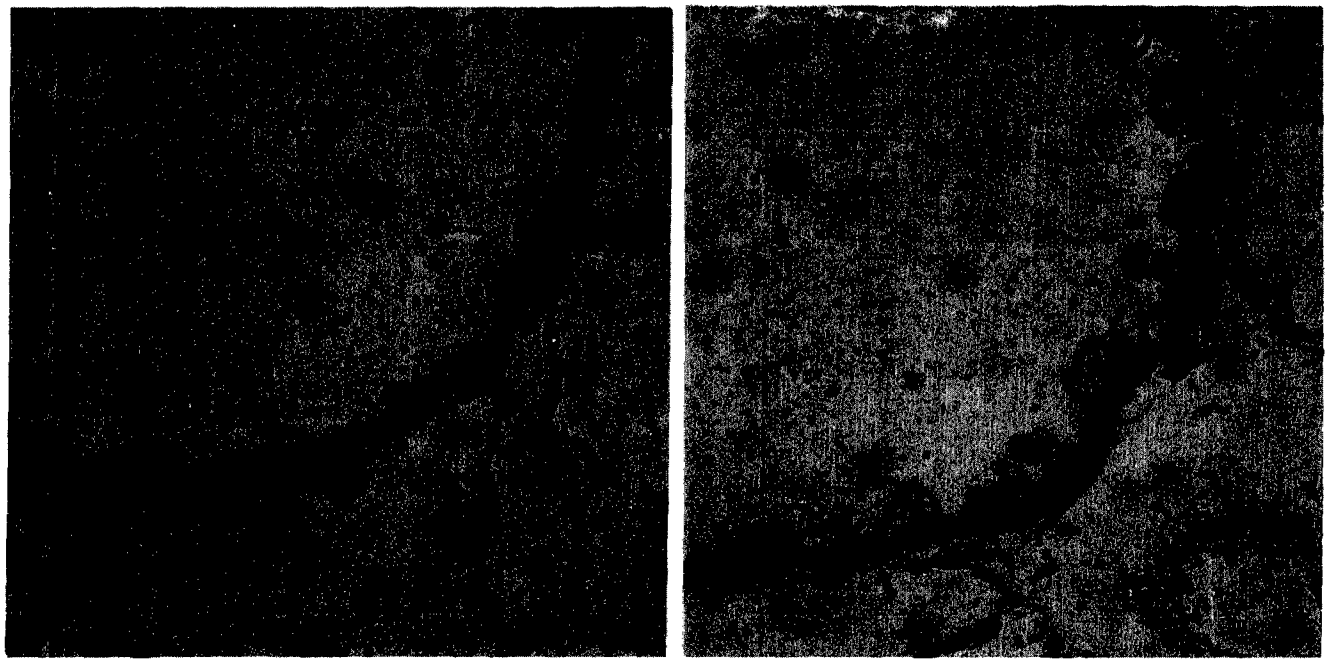


Figure 12. Light micrographs of terminal bronchiolar airways from hamsters treated with A) corn oil, or B) 800 mg/kg naphthalene 1 μ section were stained with toluidine blue. Original magnification x 400.

Male Sprague Dawley rats (163-238 g) were obtained from Hilltop Breeding Labs, Chatsworth, CA, and were divided into groups of 5 each. Naphthalene, dissolved in corn oil, was administered intraperitoneally at doses ranging from 250 mg/kg to 2000 mg/kg. Animals surviving 24 hours were sacrificed and the tissues fixed and processed as described with the hamster. As was the case in the hamster, naphthalene is acutely less toxic in the rat than in the mouse (Table 5). Examination of lung, liver and kidney sections by light microscopy revealed no apparent lesions at any of the doses tested. Thus, comparative metabolic studies in mouse and rat lung may be useful in discerning the critical molecular events leading to the pulmonary damage by naphthalene in the mouse.

TABLE 5. MORTALITY AND SITE OF TISSUE LESION AFTER IP ADMINISTRATION OF NAPHTHALENE TO RATS[a]

Dose of Naphthalene mg/kg	Mortality %	Site of Tissue Necrosis Lung	Liver	Kidney
Vehicle (corn oil)	0	0	0	0
250	0	0	0	0
500	0	0	0	0
1000	20%	0	0	0
1500	60%	0	0	0
2000	100%	0	0	0

[a] Groups of 5 male, Sprague Dawley rats were treated with naphthalene and sacrificed 24 hours later for examination of tissues by light microscopy.

COMPARISON OF COVALENT BINDING IN SUBCELLULAR FRACTIONS FROM TARGET AND NON TARGET MOUSE TISSUES

Although studies conducted thus far have demonstrated an overall correlation between the covalent binding of reactive naphthalene metabolites in mouse lung with bronchiolar damage in this tissue, high levels of binding do not necessarily lead to tissue necrosis. This raises the question of whether macromolecules to which reactive naphthalene intermediates bind differ in target and non target tissues. Thus, a study was carried out to determine whether there are differences in reactive metabolite binding to subcellular fractions from lung vs liver or kidney of the mouse.

Fifteen male Swiss Webster mice (28-38 g, Simonsen Breeding Labs, Gilroy, CA) were administered doses of ^{14}C-naphthalene (400 mg/kg, 65 dpm/nmole) intraperitoneally. Animals were sacrificed 4 hours later and lung, liver and kidney from all the animals were pooled. Microsomal, nuclear, mitochrondrial and cytosolic fractions were prepared by discontinuous sucrose gradient centrifugation as described by Mazel (1971). The pellets of each subfraction were resuspended in a small quantity of 0.25 M sucrose and aliquots of the suspension were added to 10% trichloracetic acid. The covalent binding of reactive naphthalene metabolites was assayed as described previously.

The covalent binding of radiolabel from ^{14}C-naphthalene in subcellular fractions of mouse lung, liver and kidney are presented in Table 6. In all three tissues studied, covalent binding levels in microsomal and supernatant fractions were considerably higher

TABLE 6. COVALENT BINDING OF RADIOLABEL FROM ^{14}C-NAPHTHALENE IN SUBCELLULAR FRACTIONS OF MOUSE TISSUES

Subcellular Fraction	Covalent Binding nmoles/mg Protein[a] Lung	Liver	Kidney
Homogenate	2.63	3.77	3.32
Nucleii/Debris	1.09 (41.4)	1.91 (50.7)	2.19 (66.0)
Mitochondria	1.97 (74.9)	1.90 (50.4)	1.09 (32.8)
Microsomes	3.79 (144.1)	3.57 (94.7)	2.70 (81.3)
Supernatant	3.84 (146.0)	3.98 (105.6)	3.61 (108.7)

[a] Values are the average of two determinations. Values in parenthesis indicate the covalent binding as a percentage of that in the homogenate.

than binding in mitochondrial or nuclear fractions. This was not surprising in view of the fact that reactive naphthalene metabolites probably are generated by enzymes of the smooth endoplasmic reticulum and thus the closest nucleophilic sites are in the cytosolic and microsomal fractions. A similar pattern of covalent binding in subcellular fractions has been reported for acetaminophen (Jollow, 1972). There were, however, no clear differences in the binding patterns of target (lung) vs. non target (liver, kidney) tissues.

SUMMARY AND CONCLUSIONS

Studies conducted during the past year have provided additional evidence consistent with a role of chemically reactive naphthalene metabolites in the pulmonary bronchiolar necrosis caused by this hydrocarbon. The covalent binding of reactive metabolites of naphthalene to macromolecules in lung, liver and kidney is dose related. High levels of covalent binding and bronchiolar damage are observed only at doses of 200 mg/kg and above and only after substantial depletion of tissue glutathione levels has occurred. Similar dose thresholds for covalent binding and target tissue necrosis have been observed with acetaminophen and bromobenzene. Covalent binding levels in animals treated with piperonyl butoxide prior to 400 mg/kg naphthalene were 25% of those in vehicle treated controls. Piperonyl butoxide pretreatment partially blocked glutathione depletion and decreased the bronchiolar damage by 400 mg/kg naphthalene. In comparison, SKF 525A pretreatment did not alter the glutathione depletion or lung damage by 400 mg/kg naphthalene and decreased tissue binding levels to 50% of that in corn oil plus naphthalene treated controls. Thus the levels of covalent binding in the lung appear to correlate well with the extent of pulmonary damage by naphthalene.

In comparison to vehicle treated controls, covalent binding levels and the severity of target tissues necrosis was unaltered in phenobarbital or 3-methylcholanthrene pretreated animals given 50, 100, or 200 mg/kg doses of naphthalene. Covalent binding in tissues of animals pretreated with phenobarbital and given 400 mg/kg naphthalene was 3 to 4-fold higher than in vehicle pretreated controls. However, the ratio of binding in lung to liver to kidney to muscle was similar in phenobarbital and vehicle pretreated animals given 400 mg/kg naphthalene thus indicating that there may be a common site of origin for reactive, covalently bound metabolites. Severe pulmonary bronchiolar necrosis was observed in both phenobarbital and vehicle pretreated animals given 400 mg/kg naphthalene while hepatic necrosis was noted in several phenobarbital but none of the vehicle treated animals at this naphthalene dose. Although phenobarbital and 3-methylcholanthrene pretreatments increase cytochrome P-450 monooxygenase activities they also increase the activities of the enzymes capable of detoxifying potentially reactive metabolites from naphthalene. It appears that these inducers only have an effect when all detoxifying pathways are saturated at doses above 200 mg/kg.

Large doses of p-xylene partially inactivated pulmonary cytochrome P450 monooxygenases without affecting the activity of hepatic monooxygenases in mice. P-Xylene pretreatment protected against the bronchiolar damage by 200 or 400 mg/kg doses of naphthalene but failed to selectively decrease pulmonary covalent binding by reactive naphthalene metabolites. Further studies will be necessary to determine whether this pretreatment is useful in assessing the relative roles of hepatic and extrahepatic monooxygenase metabolism of naphthalene to reactive and/or cytotoxic metabolites.

Preliminary inhalation exposures to naphthalene indicate that very small amounts of the compound given by this route may lead to severe pulmonary bronchiolar necrosis. Comparative kinetic and metabolism studies in animals treated with naphthalene by intraperitoneal and inhalation exposure are expected to provide further insights to the role of pulmonary monooxygenases in the metabolic activation and bronchiolar damage by naphthalene.

Comparative studies of naphthalene induced tissue damage conducted in the rat and hamster indicate that rat lung is unaffected even by large doses of naphthalene and hamster lung is far less sensitive than mouse lung.

Continuing comparative studies are aimed at elucidating the critical metabolic and biochemical events in the organo-selective damage by naphthalene with a view toward determining whether human lung is a sensitive or non-sensitive tissue.

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